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DNA and Chromosomes:

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The Human Tim/Tipin Complex Directly Interacts with DNA Polymerase ε and Stimulates its Synthetic Activity*

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*Running Title: Stimulation of human DNA polymerase ε activity by Tim/Tipin

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Background: The Tim/Tipin complex plays a critical role in the S phase checkpoint and replication fork stability by a molecular mechanism not yet elucidated.
Results: The human Tim/Tipin complex specifically enhances the synthetic activity of DNA polymerase ε.
Conclusion: The Tim/Tipin complex could modulate the DNA polymerase ε function at the replication fork.
Significance: These findings further our understanding of the replication fork dynamics in metazoans.

SUMMARY

The Tim/Tipin complex plays an important role in the S-phase checkpoint and replication fork stability in metazoans, but the molecular mechanism underlying its biological function is poorly understood. Herein, we present evidence that the recombinant human Tim/Tipin complex (and Tim alone) markedly enhances the synthetic activity of DNA polymerase ε. In contrast, no significant effect on the synthetic ability of human DNA polymerase α and δ by Tim/Tipin was observed. Surface plasmon resonance measurements and co-immunoprecipitation experiments revealed that recombinant DNA polymerase ε directly interacts with either Tim or Tipin. In addition, the results of DNA band shift assays suggest that the Tim/Tipin complex (or Tim alone) is able to associate with DNA polymerase ε bound to a 40-/80-mer DNA ligand. Our results are discussed in view of the molecular dynamics at the human DNA replication fork.

Accurate transfer of the genetic information is critical for survival of living organisms. Checkpoint control pathways prevent the transmission of incompletely replicated or damaged DNA to daughter cells. Genotoxic insults, spontaneous errors arising during replication as well as endogenous blocks (the so-called replication fork barriers) impede replisome progression and may trigger chromosomal rearrangements and genomic instability. To cope with these problems, cells have evolved S phase checkpoints, which sense stalled replication forks and DNA damage and delay cell cycle progression until the problems are solved [1-3].

Stabilization of stalled replication forks in the presence of DNA damage or at difficult-to-replicate templates is necessary in order to

prevent their collapse and inability to restart synthesis after recovery [4]. It was proposed that in yeast stabilization of paused forks is carried out by three proteins: Mrc1, Tof1 and Csm3 in *Saccharomyces cerevisiae* [5] and Mrc1, Swi1 and Swi3 in *Schizosaccharomyces pombe* [6]. Swi1 and Swi3 or Tof1 and Csm3 form a stable complex (*f*ork *p*rotection *c*omplex, FPC). These proteins are all mediators of the replication checkpoint [7-13] and were found to move with the replication forks in normal S phase [5, 14]. Therefore, they are thought to play a role even during unperturbed DNA replication that is independent on their checkpoint function [15]. Similar mechanisms of stabilization of the stalled forks are believed to operate also in metazoans, where Claspin, Tim and Tipin are the orthologs of Mrc1, Tof1 (Swi1) and Csm3 (Swi3), respectively [16-19]. Like the yeast counterparts, these proteins participate in S phase checkpoint acting as mediators and are associated with the replisome, as demonstrated either in human cells [19] or in *Xenopus* egg extracts [20-21]. Tim and Tipin, whose level of expression peaks at the G1/S border during the cell cycle, form a hetero-dimeric stable complex located in the nucleus [18-19]. Several studies revealed that in budding yeast Tof1 is required for the regulation of the normal progression of DNA replication [8, 12 and 15] and a reduction in the expression levels of mammalian Tim (and Tipin) resulted in a decreased rate of DNA synthesis [16-19]. Furthermore, experiments performed in the *Xenopus* egg extracts system showed that Tim and Tipin interact and collaborate with the replication factor And1 to ensure a stable association of DNA polymerase α to the replisome and that the Tim/Tipin complex is required for fork restart after aphidicolin treatment [20, 22]. This is likely due to the ability of these factors to stabilize paused replication forks and to prevent disassembly of the replisome. Consequently, forks are ready to resume DNA replication once the blockage and/or damage has been removed. Claspin, an additional important S phase checkpoint mediator, is also believed to play a critical role during unperturbed DNA replication. As a matter of fact, DNA replication in *Xenopus* egg extracts occurred somewhat more slowly than normal after immuno-depletion of Claspin [23]. Interestingly, in human cells over-expression of Claspin enhanced the rate of cell proliferation [24] and Claspin was shown to promote normal replication fork rate either in HeLa cells or in primary fibroblasts by means of DNA combing experiments [25]. In *S. cerevisiae* Mrc1 was demonstrated to directly interact with Pol2, the catalytic subunit of DNA polymerase ε, either during normal S phase or after checkpoint activation [26]. This interaction is believed to be important to avoid dissociation of DNA polymerase ε from leading strand during checkpoint. In fact, Pol2 dissociated from the replisome after HU-treatment in yeast cells lacking Mrc1 and the same behavior was also observed in Tof1-depleted cells, in line with the proposal that Mrc1 associates with Tof1 at the replication fork. Co-immunoprecipitation experiments revealed that Mrc1 interacted even with the Mcm2 subunit of the Mcm2-7 complex [26].

Overall, genetic and biochemical analyses carried out in yeasts and metazoans indicate the existence of an evolutionarily conserved molecular mechanism that functionally couples the replicative DNA helicase (the *C*dc45/*M*cm2-7/*G*INS, CMG, complex [27]) with DNA polymerase α on the lagging strand by the action of And1 and Tipin (Ctf4 in yeast) and with DNA polymerase ε on the leading strand by means of Claspin and Tim/Tipin (Mrc1 and Tof1/Csm3 or Swi1/Swi3 in budding yeast) [3]. In line with this proposal, we report here evidence that the human Tim/Tipin complex (and Tim alone) directly interacts with DNA polymerase ε and specifically enhances its synthetic activity.

EXPERIMENTAL PROCEDURES

Plasmids and baculoviruses - Human Tipin *o*pen *r*eading *f*rame (ORF) was PCR-amplified from an OriGene clone (OriGene accession number NM_017858) and cloned into the pGEX-4T-1 vector (GE Healthcare) using the following oligonucleotides: Tipin-BamHI forward (5'-TTTGGGGGATCCATGCTAGAACCACAGGAGAATGGCGTGATT-3') and Tipin-XhoI-His_6 reverse (5'-GGGTTTCATATGCTAGAACCACAGGAGAATGGCGTG-3'). The ORF sequence was confirmed by sequencing analysis (PRIMM, Milan, Italy). The plasmid expressing the truncated form of human Tim (Flag-Tim5) was already described [28, 29]. The baculoviruses expressing the human DNA polymerase ε subunits (p261, Flag-p59, p17 and p12) were a generous gift from J. Hurwitz (Memorial Sloan Kettering Cancer Center, New York, USA).

Purification of human Tipin - *Escherichia coli* Rosetta cells (Novagen) were

transformed with the plasmid pGEX-4T-1-Tipin-His_6 and grown at 37 °C in 2 litres of LB (Luria-Bertani) medium containing 100 µg/ml ampicillin and 30 µg/ml cloramphenicol. When cells reached an A_{600} of 0.8 OD, protein expression was induced by adding IPTG (at 0.2 mM) to the medium and the culture incubated for additional 4 hours at 22 °C. Cells were harvested by centrifugation (10800 x *g* for 10 minutes at 4 °C) and the resulting pellet was re-suspended in 40 ml of Tipin-lysis buffer (20 mM Hepes-NaOH, pH 7.5, 300 mM NaCl, 1% Triton X-100; 5 mM β-mercaptoethanol, 800 nM aprotinin, 4 mM benzamidine, 2 mM *p*henyl-*m*ethyl-*s*ulfonyl *f*luoride, PMSF) and subjected to three consecutive passages through a French pressure cell apparatus (Aminco Co.) at 1500 p.s.i. After centrifugation at 4 °C for 30 minutes at 65000 x *g* the cell extract was incubated with 4 ml of glutathione Sepharose beads (GE Healthcare) for 1 hour at 4 °C. Then the resin was extensively washed with of glutathione-washing buffer (20 mM Hepes-NaOH, pH 7.5, 500 mM NaCl, 1% Triton X-100, 5 mM β-mercaptoethanol) at room temperature and the protein was eluted with glutathione-elution buffer (20 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol, 20 mM L-glutathione). The fractions were pooled and incubated with thrombin protease (GE Healthcare) in a dialysis tube in order to remove the GST fused at the N-terminus of Tipin. The dialysis step was performed at room temperature for 16 hours against 3 liters of a buffer having the following composition: 20 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol. The dialyzed protein was mixed with 4 ml of glutathione Sepharose resin and incubated for 1 hour at 4 °C in order to remove the cleaved GST. The resulting flow-through fraction (which contained Tipin-His_6 but not GST) was then mixed with 4 ml of Nickel-NTA agarose resin (Qiagen) and incubated for 1 hour at 4 °C. The resin was extensively washed with Nickel-washing buffer (20 mM Hepes-NaOH, pH 7.5, 300 mM NaCl, 5 mM β-mercaptoethanol) and finally the protein was eluted with an imidazole gradient (25-500 mM) in Nickel-washing buffer. The fractions containing Tipin-His_6 were pooled and the pool was dialysed against QA buffer (20 mM Hepes-NaOH, pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol) and loaded onto a MonoQ (10/100) column at 0.5 ml/min using an ÄKTA apparatus (GE Healthcare). The column was extensively washed with the same buffer and the bound proteins were eluted with a linear gradient of NaCl (0.1-1 M). Elution fractions were collected and concentrated. 150 µg of Tipin-His_6 were loaded on a Superdex200 (10/300) column using an ÄKTA system in buffer containing 20 mM Hepes-NaOH, pH 7.5, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol. The column was calibrated by running a set of gel filtration markers that included: ferritin (440 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa) and chymotryprsinogen (25 kDa). Finally, Tipin-His_6 was concentrated and stored in aliquots at - 80 °C.

Purification of human Tim - Sf9 insect cells (20 x 10^6 cells/15-cm dish x 6 dishes) were grown at 27 °C in Sf900 medium (GIBCO) supplemented with 10% fetal bovine serum and gentamycin (10 µg/ml). The cells were infected with a fresh-amplified recombinant baculovirus stock expressing Flag-Tim. 48 hours *post*-infection cells were harvested by centrifugation at 800 x *g* for 10 min, washed with ice-cold PBS and re-suspended in 5 ml of Tim-lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4, 2 mM PMSF) containing a protease inhibitors cocktail (Roche). Cells were disrupted with 5 cycles of sonication and centrifuged at 65000 x *g* for 30 minutes at 4 °C. α-Flag M2 beads (1 ml; Sigma) were incubated with cell extract for 2 hours at 4 °C and then washed with Tim-washing buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, DTT). Bound proteins were eluted with 5 ml of Flag-elution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM DTT, 200 µg/ml Flag peptide [Sigma]). The pool of fractions containing Flag-Tim was dialyzed against 1 liter of Flag-elution buffer without Flag peptide and then concentrated up to 0.5 mg/ml. Flag-Tim (100 µg) was layered onto a 8-ml preformed glycerol gradient (from 15 to 40% glycerol in 50 mM sodium phosphate buffer, pH 7.5, 30 mM NaCl, 1 mM DTT, 0.005% Triton X-100) and subjected to centrifugation at 250000 x *g* for 12 hours at 4 °C. A parallel gradient was used to fractionate a mixture of the following standard proteins: ferritin (440 kDa), catalase (232 kDa), yeast alcohol dehydrogenase (150 kDa) and bovine serum albumin (67 kDa). Fractions containing Flag-Tim were pooled and dialyzed against the glycerol gradient buffer containing 10%

glycerol, concentrated and stored in aliquots at -80 °C.

Purification of the human Tim/Tipin complex and the truncated form Tim 5 - The human Tim/Tipin complex was purified from HEK 293T cells transiently co-transfected with plasmids expressing His_6-Tim-HA and His_6-Tipin-3xFlag, as recently described [28, 29]. Flag-Tim 5 was produced and purified using the same procedure utilized for the Tim/Tipin complex.

The human Tim/Tipin complex was also produced using the baculovirus/insect cells system Sf9 insect cells (20 x 10^6 cells/15-cm dish x 25 dishes) were grown at 27 °C in Sf900 medium (GIBCO) supplemented with 10% fetal bovine serum and gentamycin (10 µg/ml). The cells were infected with fresh-amplified recombinant baculovirus stocks expressing Flag-Tim and Tipin. 48 hours *post*-infection cells were harvested by centrifugation at 800x *g* for 10 min, washed with ice-cold PBS and re-suspended in 20 ml of Tim-lysis buffer containing a protease inhibitors cocktail (Roche). Cells were disrupted with 5 cycles of sonication and centrifuged at 65000 x *g* for 30 minutes at 4 °C. α-Flag M2 beads (1 ml) were incubated with cell extract for 2 hours at 4 °C and then washed with washing buffer Tim-washing buffer containing 2 mM PMSF. Bound proteins were eluted with 4 ml of Flag-elution buffer. The eluted sample containing the Tim/Tipin complex was dialyzed over night against buffer A (Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 0.01% Triton-X-100) and loaded onto a Mini Q 4.6/50 PE column at 0.5 ml/min using an ÄKTA apparatus (GE Healthcare). The column was extensively washed with the same buffer and the bound proteins were eluted with 8-ml linear gradient of NaCl (50 mM -1 M). Fractions (0.25 ml each) were analyzed by SDS-PAGE and the ones that contained either Tim or Tipin were dialyzed separately over night against buffer B (50 mM sodium phosphate, pH 7.5, 30 mM NaCl, 10% glycerol, 1 mM DTT, 0.005 % Triton-X-100). Aliquots of each fraction were tested for the presence of DNA polymerase ε stimulatory activity. Then, the dialyzed fractions were pooled and dialyzed over night against buffer C (50 mM sodium phosphate, pH 6.0, 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.01 % Triton-X-100). The dialyzed pool was loaded onto a Mini S 4.6/50 PE column at 0.5 ml/min using an ÄKTA apparatus (GE Healthcare). The column was extensively washed with the same buffer and the bound proteins were eluted with 5-ml linear gradient of NaCl (50 mM -1 M). Fractions (0.25 ml each) were analyzed by SDS-PAGE and the ones that contained either Tim or Tipin were dialyzed over night against buffer B (50 mM sodium phosphate, pH 7.5, 30 mM NaCl, 10% glycerol, 1 mM DTT, 0.005 % Triton-X-100). Aliquots of each fraction were tested for the presence of DNA polymerase ε stimulatory activity in enzymatic assays.

Purification of the human replicative DNA polymerases - The human DNA polymerases α and ε used for the enzymatic assays were purified from exponentially growing HeLa cells, as described [30]. DNA polymerase δ was produced in recombinant form in Sf9 insect cells and purified as described [31]. The human DNA polymerase ε used for the protein/protein interaction studies was purified from Sf9 insect cells (20 x 10^6 cells/15-cm dish x 11 dishes), which were co-infected with freshly amplified baculoviral stocks expressing its four subunits (p261, Flag-p59, p17 and p12). 48 hours post-infection cells were harvested, washed with ice-cold PBS and re-suspended in 4 ml of hypotonic buffer (20 mM Hepes-NaOH, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.01% Nonidet P-40 and 2 mM PMSF) containing a protease-inhibitors cocktail (Roche) and 30 units/ml of Benzonase (Sigma). In order to guarantee a complete cell disruption, the sample was also subjected to four sonication cycles and incubated for 30 minutes at room temperature with gentle shaking. After addition of NaCl (at 150 mM) and EDTA (at 1 mM), the sample was centrifuged at 65000x *g* for 30 minutes. The resulting supernatant was incubated with α-Flag M2 beads (1 ml) for 2 hours at 4 °C with shaking. The beads were washed with washing buffer (20 mM Hepes-NaOH, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.01% Nonidet P-40), containing 300 mM NaCl. Elution was carried out with 4 ml of the same buffer containing 150 mM NaCl at 150 mM and the Flag peptide (200 µg/ml). The fractions containing DNA polymerase ε were pooled and the pool was dialyzed against PBS buffer, concentrated and stored in aliquots at -80 °C.

DNA polymerase assays - DNA polymerase assays on poly(dA)-oligo(dT) were carried out in a volume of 25 µl using [^{3}H-Methyl]dTTP as radioactive precursor, as previously described [32]. Each DNA polymerase was titrated in the respective assay

buffer and the effect of Tim/Tipin (or Tim or Tipin alone) was measured at an amount of enzyme that gave 15-30% of the maximal activity (DNA polymerase α at 15 nM, DNA polymerase δ at 8 nM and DNA polymerase ε at 0.54 nM). The resulting level of incorporated dTTP was measured with a scintillation counter and the results were reported as the stimulation folds of each DNA polymerase against the concentrations of Tim, Tipin or Tim/Tipin used in the assays. The average values of stimulation and the standard deviations reported in the graphs were obtained from three independent experiments.

Primer extension assays were carried out using a 40-/80-mer DNA duplex as a primer/template. The 40-mer synthetic primer (5'-AGCTCCTAGGGTTACAAGCTTCACTAGG GTTGTCCTTAGG-3') was labelled at the 5'-end with [γ-^{32}P]ATP by T4 polynucleotide kinase (Roche) and purified from the unincorporated radionuclide by passage over a Micro Bio-Spin 30 column (Bio-Rad). Then, the purified primer was annealed to a 2-fold molar excess of a cold complementary 80-mer synthetic oligonucleotide that acted as the template (5'-GCTGATCAACCCTACATGTGTAGGTAAC CCTAACCCTAACCCTAAGGACAACCCTA GTGAAGCTTGTAACCCTAGGAGCT-3').
The activity of human DNA polymerase ε was titrated in mixtures (10 µl) containing the above 40-/80-mer primer/template (at 10 nM) and the four dNTPs (each at 100 nM) in the respective assay [32]. The effect of Tim/Tipin (or Tim full length and Tim 5, or Tipin alone) was analyzed using an amount of DNA polymerase ε (0.2 nM) that gave a low level of primer elongation. The reactions were carried out at 37 °C for 30 minutes, stopped with 2 µl of stop solution (98% formamide, 0.1% bromophenol blue and 0.1% xylen cyanol). The samples were denatured at 95 °C for 5 minutes, chilled on ice and then run on a 12%-polyacrilamide/bis (19:1) gel containing 7 M urea in 0.5 x TBE buffer (1× TBE had the following composition: 89 mM Tris Base, 89 mM boric acid, 2 mM EDTA, pH 8.3). Then, the gel was dried, exposed to a phosphorimaging screen and analyzed using a Typhoon Imager (GE Healthcare). The elongated primers were quantified using the ImageQuant software (GE Healthcare) and the resulting values were reported as stimulation folds of each DNA polymerase against the concentration of Tim, Tipin or Tim/Tipin used in the assays. The reported plots contain average values of stimulation with standard deviations derived from three independent experiments.

Surface plasmon resonance measurements - Dynamic interactions of the Tim and Tipin proteins with recombinant DNA polymerase ε were analyzed by using the surface plasmon resonance biosensor system Biacore2000 (Biacore). Tim (7700 *r*esonance *u*nits, RU) and Tipin (6000 RU) were immobilized on a CM5 sensor chip in 10 mM sodium acetate buffer (pH 4.0 and pH 3.5, respectively), according to the manufacturer's instructions. To collect the sensorgrams, increasing concentration of human DNA polymerase ε (1.2, 2.4, 6, 12, 24, 48 nM) in PBS buffer were fluxed over the sensor chip surface at a flow rate of 20 µl/min. Recorded sensorgrams were normalized to a baseline of 0 RU and the relative dissociation constants (K_D) values were calculated using the BIA Evaluation software (version 3.2).

Immunoprecipitation experiments - Mixtures (30 µl) containing purified Tim (2 µg) and/or recombinant DNA polymerase ε (2 µg) in PBS buffer were incubated for 1 hour at 4 °C with shaking. 30 µl of Protein A-agarose beads (Roche) were mixed with 0.6 µg of polyclonal anti-Tim antibodies (Abcam) in 300 µl of PBS buffer for 1 hour at 4 °C with shaking. After an extensive wash with PBS buffer in order to remove the unbound antibodies, the beads were re-suspended in 300 µl of PBS buffer. 100 µl of beads were mixed with the binding mixtures containing Tim and/or DNA polymerase ε and incubated for additional 2 hours at 4 °C. The beads were then washed with 3 x 600 µl of PBS buffer containing 0.1% Nonidet P-40 and re-suspended in 30 µl of SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 200 mM β-mercaptoethanol, 0.5% SDS, 0.01% blue bromophenol). Samples were subjected to electrophoresis through 8% polyacrylamide/bis (29:1) gel that was also loaded with aliquots of purified recombinant Tim and DNA polymerase ε (0.7 and 0.4 µg, respectively) and the unbound fractions (1/3 of the total).
Immuno-precipitation analyses were carried out with recombinant DNA polymerase ε (1 µg) and purified Flag-Tim 5 (0.21 µg) or full-length Flag-Tim (2 µg) in the conditions described above. Bound proteins were washed with 3 x 600 µl of PBS containing 0.2% Nonidet P-40. Aliquots of DNA polymerase ε, full-length Tim and Flag-Tim 5 (0.5, 0.35 and 0.21 µg,

respectively) and the immuno-precipitated proteins were separated through a precast denaturing gel (Any kD Mini Protean gel, Bio Rad Laboratories).
The gels were electro-blotted onto a PVDF membrane and proteins on the blot were detected with a monoclonal horseradish peroxidase-conjugated anti-Flag antibody (Abcam) or with a mouse monoclonal α-p261 antibody (Santa Cruz) using the ECL$^+$ detection system (GE Healthcare).

Immuno-precipitation analysis from cell extracts – HEK 293T cells (1.5 x 10^6/10 cm-dish) were transiently transfected with plasmids expressing full-length human Tim, the truncated versions of Tim [18] and the empty vector as a control, using the procedure previously described [28]. Cells were harvested after 18 hours *post*-transfection, washed with ice-cold PBS and re-suspended in 300 µl of IP buffer (20 mM Hepes-NaOH, pH 7.2; 150 mM KCl; 0.1% Triton X-100; 1 mM DTT; 2 mM EDTA; 1 mM Na_3VO_4; 5% glycerol, protease and phosphatase inhibitors [Roche]). Cell were disrupted with 5 cycles of sonication and centrifuged at 16000 x *g* for 30 minutes at 4 °C. Cell extracts were clarified with 10 µl of Protein G-agarose (Roche) for 1 hour at 4 °C, with shaking, and the supernatants were further incubated with 10 µl of α-Flag-agarose M2 (Sigma) for 2 hours at 4 °C. The beads were extensively washed with IP buffer and the bound proteins were re-suspended in SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 200 mM β-mercaptoethanol, 0.5% SDS, 0.01% blue bromophenol). Samples were separated through an 8% polyacrylamide/bis (29:1) gel, electro-blotted onto a polyvinylidene difluoride (PVDF) membrane and analyzed by western blot, as above described.

Electrophoretic mobility shift assays (EMSAs) - A DNA ligand made of a 40-mer DNA oligonucleotide annealed to a complementary 80-mer oligonucleotide was prepared as described above. The DNA shift assay mixtures (10 µl) had the following composition: 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.7 mM β-mercaptoethanol, 10 nM radio-labeled DNA ligand, recombinant Tim or Tipin or Tim/Tipin complex (either at 50 or 200 nM). Where indicated, DNA polymerase ε, purified from HeLa cells, was added to a final concentration of 0.1 nM. The samples were incubated for 20 minutes at 25 °C and the protein/DNA complexes were separated by electrophoresis through a 5% polyacrylamide/bis gel (37.5:1) in 0.5 × TBE buffer. Gels were dried and analyzed by phosphorimaging.

RESULTS

The Tim/Tipin complex (and Tim alone) specifically stimulates the synthetic activity of DNA polymerase ε - The Tim/Tipin complex was proposed to act as a functional tether between the replicative DNA polymerases and the DNA helicase at the replication fork. In fact, immuno-precipitation experiments carried out on extracts from synchronized HEK293T cell cultures revealed that epitope-tagged Tim and Tipin associate with the endogenous replication fork proteins, such as Mcm2 and DNA polymerase ε and δ, during S phase [19]. All that considered, we decided to investigate whether any functional interaction could take place *in vitro* between human recombinant Tim/Tipin (or the isolated Tim and Tipin proteins) and the three replicative DNA polymerases. In an initial set of experiments we analyzed whether the Tim/Tipin complex (or Tim and Tipin alone) could exert any effect on the synthetic activity of DNA polymerase α, δ and ε. The recombinant proteins used in this study are shown in Figure 1. The human Tim/Tipin complex was purified from mammalian cells transiently co-transfected with plasmids expressing His_6-Tim-HA and His_6-Tipin-3xFlag, as recently described [28-29]. A Flag-tagged version of human Tim was produced separately in insect cells infected with a recombinant baculovirus; whereas, human Tipin was expressed separately in bacterial cells as a fusion with GST at the N-terminus (cleavable with the site-specific endo-protease thrombin) and a poly-Histidine tag at the C-terminus and purified as described in *Experimental Procedures*. After having titrated the synthetic activity of the human replicative DNA polymerases using poly(dA)-oligo(dT) as a primer/template in the specific reaction conditions, we found that the Tim/Tipin complex and Tim alone could dose-dependently and specifically stimulate the synthetic activity of DNA polymerase ε, as shown in Figure 2. We observed that incorporation of radio-labelled dTTP by DNA polymerase ε was increased by more than 15 folds in the presence of Tim/Tipin at concentrations that were above 100 nM. These values represent a molar excess of more than 200 folds over the DNA polymerase concentration used in the assays (0.6 nM). The high molar ratio between Tim/Tipin (or Tim

alone) and DNA polymerase ε required for the maximal stimulation might depend on the fact that only a fraction of the recombinant Tim/Tipin complex retains a putative cell cycle-specific post-translational modification required for its full biological activity. On the other hand, we observed a modest stimulation of DNA polymerase δ and α synthetic activity in the presence of the Tim/Tipin complex (or Tim alone). No effect was detected on all the three replicative DNA polymerases by increasing amounts of the purified recombinant Tipin protein. Moreover, Tim/Tipin (or Tim alone) did not affect the synthetic function of DNA polymerase δ also in the presence of the sliding clamp Proliferating Cell Nuclear Antigen (PCNA; V. Aria *et al.*, data not shown). Next we carried out an additional set of assays in order to visualize the products synthesized by the DNA polymerases in the presence of Tim/Tipin (or Tim alone). This analysis was performed using a DNA duplex made of a 40-mer primer annealed to a complementary 80-mer template as the substrate. As shown in Figure 3, we found that the synthetic activity of DNA polymerase ε was markedly enhanced on this primer/template in the presence of Tim/Tipin (up to 15 folds). Instead, no effect was detected on DNA polymerase α and δ on the same substrate (V. Aria *et al.*, data not shown) even when we carried out the enzymatic assays at the same polymerase:Tim molar ratio used to obtain the maximal stimulation of the DNA polymerase ε activity (V. Aria *et al.*, data not shown). To rule out the possibility that our purified samples of Tim/Tipin (and Tim alone) were contaminated by any polymerase activity, in all sets of experiments we performed control assays where the maximal amount of these proteins was used without adding the tested DNA polymerase (i. e., see *lanes 10* and *18* of the gel in Figure 3A). It could be also questioned that the stimulation of DNA polymerase ε is not due to the Tim/Tipin complex but to any contaminating protein from the HeLa cell extract that remains associated to it during the purification procedure. To address this issue, we produced human Tim/Tipin using the insect cell-baculovirus expression system, purified the recombinant protein complex by a procedure that, in addition to the Flag-agarose affinity step, included two high-resolution chromatographies on ion-exchange columns (Mini Q and Mini S) and found that the DNA polymerase ε stimulatory activity tracked with the Tim/Tipin protein peaks eluted from these columns with salt gradients (Figure 4).

In order to analyze the effect of Tim/Tipin on the polymerase processivity we carried out assays at a low enzyme to primer/template ratio (1:20) for shorter incubation times to ensure that each product would correspond to a single DNA polymerase ε-DNA elongation event (single-hit conditions; [33]). This analysis revealed that in the presence of Tim/Tipin (and Tim alone) length distribution of the products synthesized by DNA polymerase ε did not change (V. Aria *et al*., data not shown).

Taken together, these data suggest that the Tim/Tipin complex (or Tim alone) enhances the synthetic function of DNA polymerase ε in a dose-dependent and specific manner on various kinds of DNA primer/template molecules, whereas Tipin alone does not exert any stimulatory effect on any of the three replicative DNA polymerases.

Tim and Tipin directly interact with DNA polymerase ε - The finding that Tim/Tipin (or Tim alone) specifically stimulated DNA polymerase ε prompted us to investigate whether a direct physical interaction could be detected between these proteins *in vitro* by using the surface plasmon resonance technique. For these analyses the hetero-tetrameric DNA polymerase ε (purified from baculovirus-infected insect cells; Figure 1, *lane 5*) was fluxed over a sensor-chip on which recombinant Tim (or Tipin) was immobilized. Figure 5 shows the overlaid sensorgrams obtained by testing six increasing concentrations of human DNA polymerase ε (from 1 to 50 nM) either on Tim- or Tipin-immobilized sensor-chips (*Panel A* and *B*, respectively). These results indicated that DNA polymerase ε physically interacted with both Tim and Tipin, although with different binding affinity (K_D of 6.8 x 10^{-8} M for Tim and 5.3 x 10^{-6} M for Tipin). These data were confirmed by co-immuno-precipitation of the recombinant proteins (Figure 6). In these experiments we used Protein A-agarose beads conjugated with anti-Tim antibodies on mixtures containing recombinant hetero-tetrameric DNA polymerase ε (with a Flag-tagged version of the p59 subunit) and Flag-tagged Tim. The results of this analysis revealed that the recombinant human DNA polymerase ε directly interacted with Tim.

Next we analyzed the physical interaction of DNA polymerase ε with many Tim truncated forms already described [18]. Plasmids expressing Flag-tagged versions of

these Tim truncated forms were transiently transfected into HEK 293T cells. Interaction of these proteins with the endogenous DNA polymerase ε was tested by co-immuno-precipitation experiments carried out on extracts of the transfected cells. Flag-Tim 5 (which includes residues 1082-1208 of Tim polypeptide chain) was found to be unable to interact with the endogenous DNA polymerase ε, whereas all the other tested Tim deleted forms co-immuno-precipitated with DNA polymerase ε although to a different extent (Figure 7). These findings suggest that an extended surface of the Tim polypeptide chain might be involved in the interaction with DNA polymerase ε. Then, we purified the Flag-Tim 5 protein from HEK 293T transfected cells by the same procedure used for the Tim/Tipin complex. We found that Flag-Tim 5 did not directly interact with human recombinant DNA polymerase ε, as indicated by co-immuno-precipitation assays with anti-Tim antibodies (Figure 8, *Panel A*). Besides, Flag-Tim 5 was unable to enhance the synthetic activity of DNA polymerase ε on a 40-mer/80-mer primer/template (Figure 8, *Panel B*). These results provide further evidence that the observed stimulatory effect on DNA polymerase ε is truly due to Tim/Tipin and not to any contaminating activity of our protein preparations.

DNA binding activity of Tim/Tipin and DNA polymerase ε - We analyzed whether DNA polymerase ε affected DNA binding of the Tim/Tipin complex and Tim and Tipin alone. It was previously observed that *S. cerevisiae* DNA polymerase ε prefers primer/template DNA molecules having a duplex region of at least 40 base pairs as a substrate in enzymatic assays *in vitro* [33]. Based on this observation, we performed band-shift assays of human DNA polymerase ε in the absence and presence of Tim/Tipin (or Tim and Tipin alone) on the same 40-/80-mer utilized as the primer/template in the polymerase assays (Figure 9). A fixed amount of DNA polymerase ε (0.1 nM) was used in the presence of two amounts of Tim/Tipin (and Tim or Tipin alone). We found that DNA polymerase ε alone (*lane 2* in each gel) formed a single protein/DNA complex. Addition of Tim/Tipin (at 50 and 200 nM in *lane 3* and *4*, respectively, of the gel shown in *Panel A* of Figure 9) resulted in the formation of a super-shifted band. This is likely to correspond to a DNA-DNA polymerase ε-Tim/Tipin ternary complex, because the same amounts of Tim/Tipin in the absence of DNA polymerase ε did not produce any appreciable DNA-shift (see *lanes 6* and *7* in Figure 9 *Panel A*). Similarly, in the presence of Tim a super-shifted band was detected in addition to the one due to the DNA binding activity of DNA polymerase ε alone (*lanes 2-4* in the gel reported in Figure 9 *Panel B*). This is likely to correspond to a ternary complex containing DNA, DNA polymerase ε and Tim, since the same amounts of Tim did not result in any appreciable DNA-shift, when DNA polymerase ε was absent (*lanes 6* and *7* in the gel in Figure 9 *Panel B*). In contrast, in similar assays Tipin did not bind DNA either alone or in the presence of DNA polymerase ε (see gel shown in Figure 9, *Panel C*). Overall, these EMSAs suggested that Tim/Tipin and Tim alone interacted with DNA polymerase ε bound to a 40-/80-mer DNA primer/template.

DISCUSSION

Replication checkpoint pathways respond to DNA damage and to various stress conditions during S phase by delaying cell cycle progression, stabilizing stalled forks and promoting DNA repair. An evolutionarily conserved set of proteins (Tof1/Swi1/Tim, Csm3/Swi3/Tipin, Ctf4/Mcl1/And1 and Mrc1/Claspin) plays a critical role in this process. It moves with the replication forks in normal S-phase both in yeasts and in metazoans and is thought to stabilize the replisome even during unperturbed replication in a way that is independent of its checkpoint function [5, 14, 19]. This mechanism reduces the extension of single-stranded DNA regions due to the uncoupling of the replicative DNA polymerases from the DNA unwinding machinery at the paused replication forks and ensures that components of the replisome are hold together ready to resume their activity (DNA synthesis and unwinding) after the obstacle has been removed [3]. Coordination of the replicative DNA polymerases and the DNA helicase (CMG complex [27]) takes place even during normal S phase and in the absence of these proteins all replication forks move at only half the normal rate in yeast cells [12, 15].

In *Xenopus laevis* and in human cells Tim and Tipin were demonstrated to be replication fork-associated factors, but their function is not essential for genome duplication [19-21]. Nonetheless, these proteins appeared to be critical for efficient cell growth and/or organism development, since knockout of mouse Tim provoked embryonic lethality [34], whereas

its down-regulation affected the correct lung and kidney morphogenesis [35-36]. The exact biochemical functions played by Tim, Tipin and/or the Tim/Tipin complex in mammals has not been elucidated so far, although they are clearly implicated in S-phase checkpoint, DNA replication and in chromosome cohesion [16-19, 37-38]. Furthermore, while a number of genetic interactions were described in yeast for Tof1/Swi1 and Csm3/Swi3, only a few direct protein/protein interactions involving the mammalian counterparts of these factors were identified so far. In fact, human Tipin was demonstrated in two-hybrid screens to directly interact with the 34-kDa subunit of replication protein A and peroxiredoxin2 [19], and *Xenopus* Tipin was found to directly interact with the replication/cohesion factor And1 (Ctf4 in *S. cerevisiae*) and the p180 catalytic subunit of DNA polymerase α [22]. On the other hand, only Claspin, in addition to Tipin, was reported so far to be a direct binding partner of Tim [39].

Our analysis revealed that the human Tim/Tipin complex (and Tim alone) was able to enhance the synthetic activity of DNA polymerase ε *in vitro* in the absence of other replication factors (such as replication protein A, replication factor C and proliferating cell nuclear antigen). Therefore, the observed stimulation appears to derive from a direct action of Tim/Tipin (or Tim alone) on DNA polymerase ε without the intervention of additional replication factors. In fact, we demonstrated that both Tim and Tipin directly interacted with DNA polymerase ε by surface plasmon resonance measurements and by co-immuno-precipitation experiments. Furthermore, the results of EMSAs suggested that the Tim/Tipin complex (and Tim alone, even though to a lesser extent) associated with DNA polymerase ε bound to DNA, as revealed by the presence of a super-shifted DNA band when Tim/Tipin (or Tim alone) was added to the mixtures (Figure 9). In addition, we believe unlikely that Tim/Tipin and Tim alone stimulated DNA polymerase ε in an unspecific way, simply by covering inhibitory sites on the single-stranded DNA template. In fact, we found that both Tim/Tipin and Tim alone bound the 40-/80-mer primer/template with very low affinity in EMSAs (Figure 9 and our unpublished results) and, moreover, the stimulatory effect was exerted specifically on DNA polymerase ε, but not on the two other replicative DNA polymerases.

The results of this work suggest that the stimulation of human DNA polymerase ε by Tim/Tipin (or Tim alone) does not derive from an increase of the enzyme processivity and we can postulate that it could be due to an improved primer/template utilization whose molecular bases require a more thorough investigation to be clarified. However, it is worth noting that this effect was specifically observed for DNA polymerase ε, the enzyme acting on the leading strand, whereas the synthetic activity of the lagging-strand DNA polymerases (DNA polymerase α and δ) was not affected in the presence of Tim/Tipin (or either Tim or Tipin alone). It should be mentioned that the yeast Mrc1 factor was found to directly interact with the catalytic subunit of DNA polymerase ε [26] and to stably associate with Tof1/Csm3 (and Swi1/Swi3) [5-6]. Furthermore, human Claspin was found to stably associate with the replisome and to interact with Tim and DNA polymerase ε [28, 39]. On the other hand, in the *Xenopus* egg extracts system and in mammalian cells, it was demonstrated that Tipin and the replication factor And1 (Ctf4 in yeast) interact with each other and independently bind the p180 catalytic subunit of DNA polymerase α to facilitate its loading and stable association with chromatin [22, 40]. Our unpublished results indicated that the Tim/Tipin complex directly interacted with recombinant purified Mcm2-7 complex and Cdc45, which are components of the CMG complex, the replicative DNA helicase [27]. The yeast and *Xenopus* counterparts of these factors together with Mrc1/Claspin were found to associate with the advancing replisome and to interact *in vivo* with the CMG complex [14, 20, 26].

Overall, biochemical analyses carried out in yeasts and metazoans suggest the existence of an evolutionarily conserved mechanism to ensure replication fork stability and coordination of DNA unwinding and synthesis. This mechanism is based on the interaction of Mrc1/Claspin and Tof1/Swi1/Tim with DNA polymerase ε on the leading strand and of Ctf4/Mcl1/And1 and Csm3/Swi3/Tipin with DNA polymerase α on the lagging strand [3]. It would be interesting to analyze whether Claspin in collaboration with Tim/Tipin affects the synthetic activity of the replicative DNA polymerases and/or the unwinding function of the CMG complex. In fact, Claspin is an additional component of the multi-molecular assembly responsible for the safeguard of the

replisome and coordination of its enzymatic functions.

REFERENCES

1. Melo, J., and Toczyski, D. (2002) A unified view of the DNA damage checkpoint. *Curr. Opin. Cell Biol*., **14**, 237-245.
2. Branzei, D. and Foiani, M. (2005) The DNA damage response during DNA replication. *Curr. Opin. Cell Biol.*, **17**, 568-575.
3. Errico, A. and Costanzo, V. (2012) Mechanisms of replication fork protection: a safeguard for genome stability. *Crit. Rev. Biochem. Mol. Biol*., **47**, 222-235.
4. Tercero, J. A., Longhese, M. P. and Diffley, J. F. (2003) A central role for DNA replication forks in checkpoint activation and response. *Mol. Cell*, **11**, 1323-1336.
5. Bando, M., Katou, Y., Komata, M., Tanaka, H., Itoh, T., Sutani, T. and Shirahige, K. (2009) Csm3, Tof1 and Mrc1 form a hetero-trimeric mediator complex that associates with DNA replication forks. *J. Biol. Chem.,* **284**, 34355-34365.
6. Tanaka, T., Yokoyama, M., Matsumoto, S., Fukatsu, R., You, Z. and Masai, H. (2010) Fission yeast Sw1-Swi3 complex facilitates DNA binding of Mrc1. *J. Biol. Chem.*, **285**, 39609-39622.
7. Noguchi, E., Noguchi, C., McDonald, W. H., Yates, J. R., 3rd and Russell, P. (2004) Swi1-Swi3 are components of a replication fork protection complex in fission yeast. *Mol. Cell. Biol.*, **24**, 8342-8355.
8. Katou, Y., Kanoh, Y., Bando, M., Noguchi, H., Tanaka, H., Ashikari, T., Sugimoto, K. and Shirahige, K. (2003) S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature*, **424**, 1078-1083.
9. Nedelcheva, M. N., Roguev, A., Dolapchiev, L. B., Shevchenko, A., Taskov, H. B., Shevchenko, A., Stewart, A. F. and Stoynov, S. S. (2005) Uncoupling of unwinding from DNA synthesis implies regulation of MCM helicase by Tof1/Mrc1/Csm3 checkpoint complex. *J. Mol. Biol*., **347**, 509-521.
10. Tanaka, K., and Russell, P. (2001) Mrc1 channels the DNA replication arrest signal to checkpoint kinase Cds1. *Nat. Cell Biol*., **3**, 966-972.
11. Foss, E. J. (2001) Tof1p regulates DNA damage responses during S phase in *Saccharomyces cerevisiae*. *Genetics*, **157**, 567-577.
12. Tourriere, H., Versini, G., Cordon-Preciado, V., Alabert, C. and Pasero, P. (2005) Mrc1 and Tof1 promote replication fork progression and recovery independently of Rad53. *Mol. Cell*, **19**, 699-706.
13. Calzada, A., Hodgson, B., Kanemaki, M., Bueno, A. and Labib, K. (2005) Molecular anatomy and regulation of a stable replisome at a paused eukaryotic DNA replication fork. *Genes Dev*. **19**, 1905-1919.
14. Gambus, A., Jones, R. C., Sanchez-Diaz, A., Kanemaki, M., van Deursen, F., Edmondson, R. D. and Labib, K. (2006) GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nat. Cell Biol*., **8**, 358-366.
15. Hodgson, B., Calzada, A. and Labib, K. (2007) Mrc1 and Tof1 regulate DNA replication forks in different ways during normal S phase. *Mol Cell. Biol.*, **18**, 3894-3902.
16. Chou, D. M. and Elledge, S. J. (2006) Tipin and Timeless form a mutually protective complex required for genotoxic stress resistance and checkpoint function. *Proc. Natl. Acad. Sci. USA*, **103**, 18143-18147.
17. Unsal-Kacmaz, K., Chastain, P. D., Qu, P. P., Minoo, P., Cordeiro-Stone, M., Sancar, A. and Kaufmann, W. K. (2007) The human Tim/Tipin complex coordinates an intra-S checkpoint response to UV that slows replication fork displacement. *Mol. Cell Biol*., **27**, 3131-3142.
18. Yoshizawa-Sugata, N. and Masai, H. (2007) Human Tim/Timeless-interacting protein, Tipin, is required for efficient progression of S phase and DNA replication checkpoint. *J. Biol. Chem*., **282**, 2729-2740.
19. Gotter, A. L., Suppa, C. and Emanuel, B. S. (2007) Mammalian TIMELESS and Tipin are evolutionarily conserved replication fork-associated factors. *J. Mol. Biol*., **366**, 36-52.

20. Errico, A., Costanzo, V. and Hunt, T. (2007) Tipin is required for stalled replication forks to resume DNA replication after removal of aphidicolin in *Xenopus* egg extracts. *Proc. Natl. Acad. Sci. USA*, **104**, 14929-14934.
21. Lee, J., Gold, D. A., Shevchenko, A., Shevchenko, A. and Dunphy, W. G. (2005) Roles of replication fork-interacting and Chk1-activating domains from Claspin in a DNA replication checkpoint response. *Mol. Biol. Cell*, **16**, 5269-5282.
22. Errico, A., Cosentino, C., Rivera, T., Losada, A., Schwob, E., Hunt. T. and Costanzo, V. (2009) Tipin/Tim/And1 protein complex promotes Pol alpha chromatin binding and sister chromatid cohesion. *EMBO J.*, **28**, 3681-3692.
23. Lee, J., Kumagai, A. and Dunphy, W. G. (2003) Claspin, a Chk1-regulatory protein, monitors DNA replication on chromatin independently of RPA, ATR, and Rad17. *Mol. Cell*, **11**, 329-340.
24. Lin, S. Y., Li, K., Stewart, G. S. and Elledge, S. J. (2004) Human Claspin works with BRCA1 to both positively and negatively regulate cell proliferation. *Proc. Natl. Acad. Sci. USA*, **101**, 6484-6489.
25. Petermann, E., Helleday, T. and Caldecott, K. W. (2008) Claspin promotes normal replication fork rates in human cells. *Mol. Biol. Cell*, **19**, 2373-2378.
26. Lou, H., Komata, M., Katou, Y., Guan, Z., Reis, C. C., Budd, M., Shirahige, K. and Campbell, J. L. (2008) Mrc1 and DNA polymerase epsilon function together in linking DNA replication and S phase checkpoint. *Mol. Cell*, **32**, 106-117.
27. Ilves, I., Petojevic, T., Pesavento, J. J. and Botchan, M. R. (2010) Activation of the MCM2-7 helicase by association with Cdc45 and GINS proteins. *Mol. Cell*, **37**, 247-258.
28. Uno, S. and Masai, H. (2011) Efficient expression and purification of human replication fork-stabilizing factor, Claspin, from mammalian cells: DNA-binding activity and novel protein interactions. *Genes Cells*, **16**, 842-856.
29. Uno, S., You, Z. and Masai, H. (2012) Purification of replication factors using insect and mammalian cell expression systems. *Methods*, **57**, 214-221.
30. Syväoja, J., Suomensaari, S., Nishida, C., Goldsmith, J. S., Chui, G. S. J., Jain, S. and Linn, S. (1990) DNA polymerases alpha, delta, and epsilon: three distinct enzymes from HeLa cells. *Proc. Natl. Acad. Sci. USA*, **87**, 6664-6668.
31. Podust, V. N., Chang, L.S., Ott, R., Dianov, G. L. and Fanning. E. (2002) Reconstitution of human DNA polymerase delta using recombinant baculoviruses: the p12 subunit potentiates DNA polymerizing activity of the four-subunit enzyme. *J. Biol. Chem.*, **277**, 3894-3901.
32. Weiser, T., Gassmann, M., Thommes, P., Ferrari, E., Hafkemeyer, P. and Hübscher, U. (1991) Biochemical and functional comparison of DNA polymerases alpha, delta, and epsilon from calf thymus. *J. Biol. Chem*., **266**, 10420-10428.
33. Asturias, F. J., Cheung, I. K., Sabouri, N., Chilkova, O., Wepplo, D. and Johansson, E. (2006) Structure of *Saccharomyces cerevisiae* DNA polymerase epsilon by cryo-electron microscopy. *Nature Struct. Mol. Biol.*, **13**, 35-43.
34. Gotter, A. L., Manganaro, T., Weaver, D. R., Kolakowski, L. F. Jr, Possidente, B., Sriram, S. *et al.* (2000) A time-less function for mouse Timeless. *Nature Neurosci*., **3**, 755-756.
35. Li, Z., Stuart, L. O., Qiao, J., Pavlova, A., Bush, K. T., Pohl, M. *et al.* (2000) A role for Timeless in epithelial morphogenesis during kidney development. *Proc. Natl. Acad. Sci. USA,* **97**, 10038-10043.
36. Xiao, J. Li, C., Zhu, N. L., Borok, Z. and Minoo, P. (2003) Timeless in lung morphogenesis. *Dev. Dynam*., **228**, 82-94.
37. Chan, R. C., Chan, A., Jeon, M., Wu, T. F., Pasqualone, D., Rougvie, A. E. and Meyer, B. J. (2003) Chromosome cohesion is regulated by a clock gene paralogue TIM-1. *Nature*, **423**, 1002-1009.
38. Smith-Roe, S. L., Patel, S. S., Simpson, D. A., Zhou, Y. C., Rao, S., Ibrahim, J. G., Kaiser-Rogers, K. A., Cordeiro-Stone, M. and Kaufmann, W. K. (2011) Timeless functions independently of the Tim-Tipin complex to promote sister chromatid cohesion in normal human fibroblasts. *Cell Cycle*, **10**, 1618-1624.
39. Serçin, Ö. and Kemp, M. K. (2011) Characterization of functional domains in human Claspin. *Cell Cycle*, **10**, 1-8.

40. Zhu, W., Ukomadu, C., Jha, S., Senga, T., Dhar, S. K., Wohlschlegel, J. A., Nutt, L. K., Kornbluth, S. and Dutta, A. (2007) Mcm10 and And-1/CTF4 recruit DNA polymerase alpha to chromatin for initiation of DNA replication. *Genes Dev.*, **21**, 2289-2299.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Purified recombinant proteins. His_6-Tim-HA/His_6-Tipin-3Flag complex (5 μg, *lane* 1), Flag-Tim (4 μg, *lane* 2), Tipin-His_6 (5 μg, *lane* 3, extra bands are due to proteolytic degradation of the protein) and DNA polymerase ε complex (10 μg, *lane 4*) were run on a 4-15% polyacrylamide gradient-SDS gel, which was stained with Coomassie Blue. The DNA polymerase ε subunits (p261, Flag-p59, p17 and p12) are indicated on the *right*. Migration of marker proteins run on a parallel lane is indicated on the *left*.

Figure 2. Effect of human Tim/Tipin, Tim and Tipin on replicative DNA polymerases synthetic activity. The amount of [^{3}H-Methyl]dTTP incorporated into a poly(dA)-oligo(dT) substrate by DNA polymerase α (15 nM, *Panel A*), δ (8 nM, *Panel B*) and ε (0.54 nM, *Panel C*) were analyzed in the presence of increasing concentration of Tim/Tipin (*circles*) or Tim alone (*squares*) or Tipin alone (*diamonds*). The activities were normalised to the values calculated in the absence of Tim/Tipin, Tim and Tipin alone.

Figure 3. Primer elongation capability of DNA polymerase ε is enhanced by Tim/Tipin and Tim. (A) Elongation activity of DNA polymerase ε was measured on a 40-/80-mer as the primer/template, as described in *Experimental Procedures*. A fixed amount of DNA polymerase ε (0.2 nM) was assayed alone (*lane 2*) or in the presence of increasing concentrations of recombinant Tim or Tim/Tipin complex (12.5, 25, 50, 100, 200, 400, 700 nM; *lanes 3-9* and *lanes 11-17*, respectively). Control assays were carried out which contained only Tim or Tim/Tipin (at 700 nM) in the absence of DNA polymerase ε (*lanes 10* and *18*, respectively). All the reactions, including the blank sample (*lane 1*), were stopped and loaded on a 12% polyacrylamide/bis (19:1) gel containing 7 M urea in 0.5 x TBE buffer. The electrophoretic run was carried out in the same buffer at 30 Watt. (B) The radioactivity of the elongated products was quantified using the ImageQuant software (GE Healthcare). The intensity of the signal in each lane was normalized to the value calculated for the reaction carried out by DNA polymerase ε alone. In the plot average values are reported with error bars from three independent experiments carried out in the presence of Tim (*squares*) or Tim/Tipin (*circles*).

Figure 4. Analysis of the recombinant human Flag-Tim/Tipin complex purified from insect cells. An aliquot of the indicated fractions eluted with a salt gradient from Mini Q (20 μl; A) and Mini S (10 μl; B) columns were analyzed by a denaturing 8% polyacrylamide:bis (29:1) gel stained with Coomassie Blue. Migration of marker proteins run on a parallel lane is indicated on the *left*. An aliquot (2 μl) of

the indicated fractions eluted from Mini Q (C) or Mini S (D) was analyzed for the presence of DNA polymerase ε stimulatory activity using a 40-/80-mer as the primer/template, as described in the text. The assays were carried out in the presence of a fixed amount of DNA polymerase ε (*Panel C*, *lanes 2-9*; *Panel D*, *lanes 2-7*) or without DNA polymerase ε as control reactions (*Panel C, lanes 10-16*; *Panel D*, *lanes 8-12*). All the reactions, including the blank sample (*lane 1* of each gel), were stopped and loaded on a 12% polyacrylamide/bis (19:1) gel containing 7 M urea in 0.5 x TBE buffer. The electrophoretic run was carried out in the same buffer at 30 Watt. The radioactivity of the elongated products was quantified using the ImageQuant software (GE Healthcare). The intensity of the signal in each lane was normalized to the value calculated for the reaction carried out by DNA polymerase ε alone.

Figure 5. Dynamic interaction of DNA polymerase ε with immobilized Tim and Tipin. The recombinant DNA polymerase ε complex was fluxed at increasing concentrations (1.2, 2.4, 6, 12, 24 and 48 nM**,** *lower* to *upper curves*) over a Tim- and Tipin-immobilized sensor-chip using a Biacore 2000 instrument (*Panel A* and *B*, respectively). An overlaid plot of sensorgrams was obtained corresponding to the recorded resonance units (RU) versus time (s).

Figure 6. DNA polymerase ε and Tim co-immunoprecipitate. Immuno-precipitation experiments were carried out using Protein A-agarose beads conjugated with anti-Tim antibodies and mixtures of purified recombinant Flag-Tim and DNA polymerase ε, as described in *Experimental Procedures*. The samples were subjected to SDS-PAGE and Western blot analysis with anti-Flag antibodies to detect recombinant Flag-Tim and the Flag-p59 subunit of the recombinant DNA polymerase ε, as indicated. Aliquots of Flag-Tim and DNA polymerase ε (0.7 and 0.4 μg, *lanes* 1 and 2, respectively) as input, the bound (*lanes 3-5*) and unbound proteins (*lanes 6-8*) present in each sample were analyzed.

Figure 7. Interaction of Tim truncated forms expressed in mammalian cells with endogenous DNA polymerase ε. (A) Extracts of HEK 293T cells not transfected (*lane 1*), transfected with empty vector (*lane 2*) or with plasmids expressing full-length Flag-Tim or its truncated derivatives (*lanes 3-8*) were subjected to co-immuno-precipitation experiments with α-Flag-agarose beads, as indicated in *Experimental Procedures*. Bound proteins were analyzed by immuno-blotting and detected with monoclonal α-Flag (Abcam) and α-p261 (Santa Cruz) antibodies. (B) Schematic representations of the Tim truncated forms used in *Panel A*, as previously described [18].

Figure 8. Physical and functional interaction of purified Tim 5 with DNA polymerase ε. (A) Immuno-precipitation experiments were carried out on mixture of purified DNA polymerase ε, full-length Tim or Tim 5 with α-Tim antibodies, previously immobilized on Protein A-agarose, as indicated in *Experimental Procedures.* DNA polymerase ε (0.5 μg), full-length Tim (0.35 μg) and Tim 5 (0.21 μg) and bound proteins (*lanes 1-8*) were analyzed by Western blot using α-Flag antibodies. (B) The elongation capability of a fixed amount of DNA polymerase ε (0.2 nM) alone (*lane 2*) or in the presence of increasing concentrations of purified Tim 5 (12.5, 25, 50, 100, 200, 400, 700 nM, *lanes 4-10*, respectively) or Tim/Tipin (700 nM, *lane 11*) was measured on a 40-/80-mer substrate, as indicated in *Experimental Procedures*. Control assays were carried out with Tim 5 alone (700 nM, *lane 3*).

Figure 9. EMSAs with DNA polymerase ε and Tim/Tipin (and Tim or Tipin alone). DNA-band shift assays were carried out using a radio-labelled 40-/80-mer DNA ligand (10 nM) and DNA polymerase ε either alone (*lane 2* of each gel) or in the presence of the indicated concentrations of Tim/Tipin, Tim and Tipin alone (*lanes 3* and *4* of *Panels A-C*, respectively). The DNA binding activity of Tim/Tipin, Tim and Tipin alone was also assayed (*lanes 5* and *6* of each gel). Samples were run on native polyacrylamide/bis (37.5:1) gels in 0.5 x TBE. After electrophoresis gels were dried and analyzed by phosphorimaging, as described in *Experimental Procedures*. Unbound DNA, wells and protein-DNA complexes are indicated on the *right side* of each gel.

Fig. 1

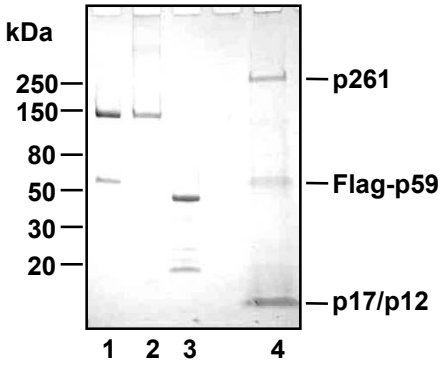


Fig. 2

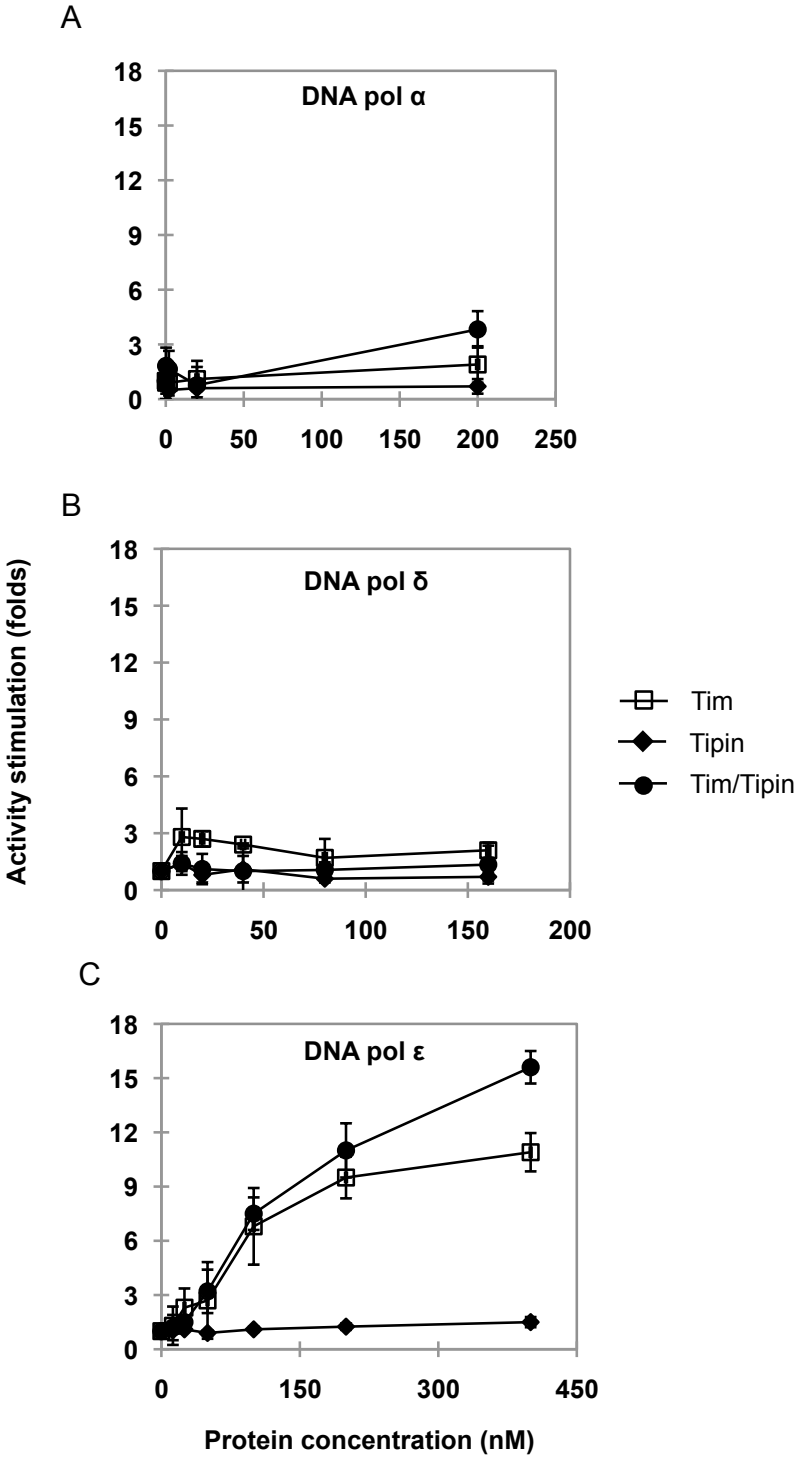


Fig. 3

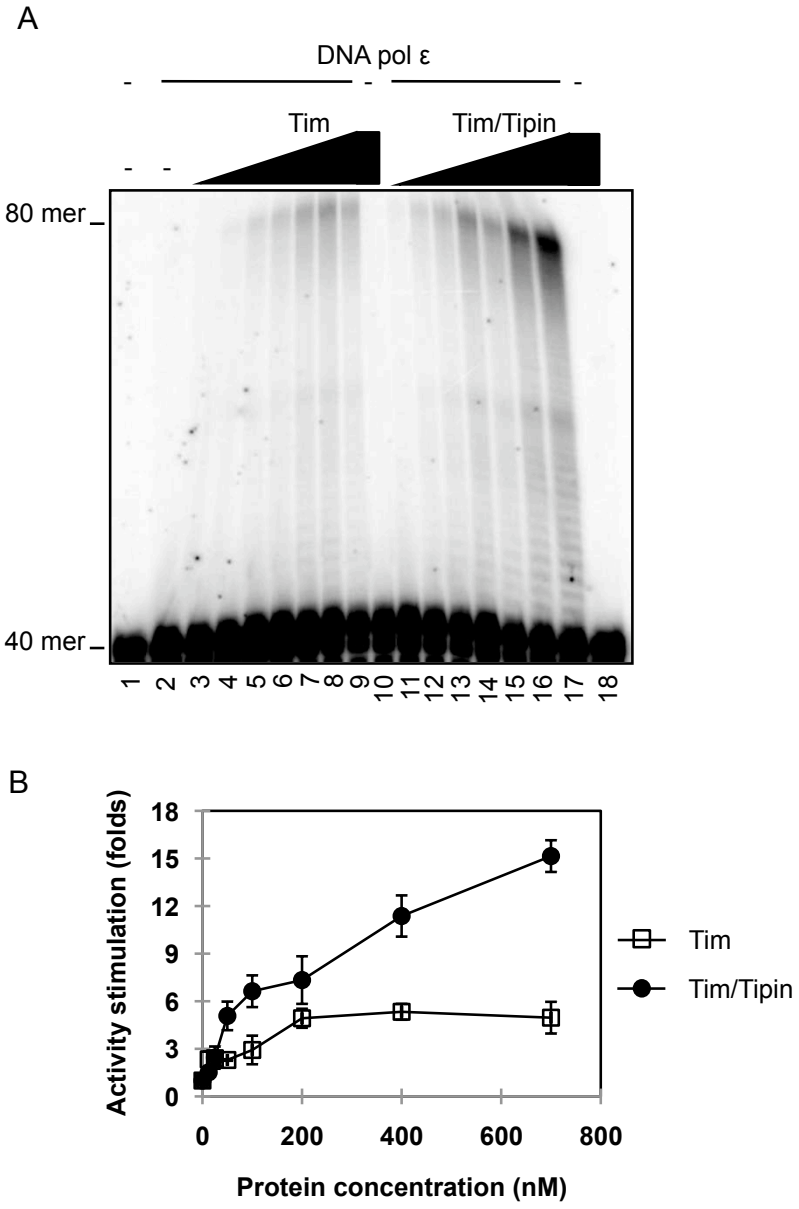


Fig. 4

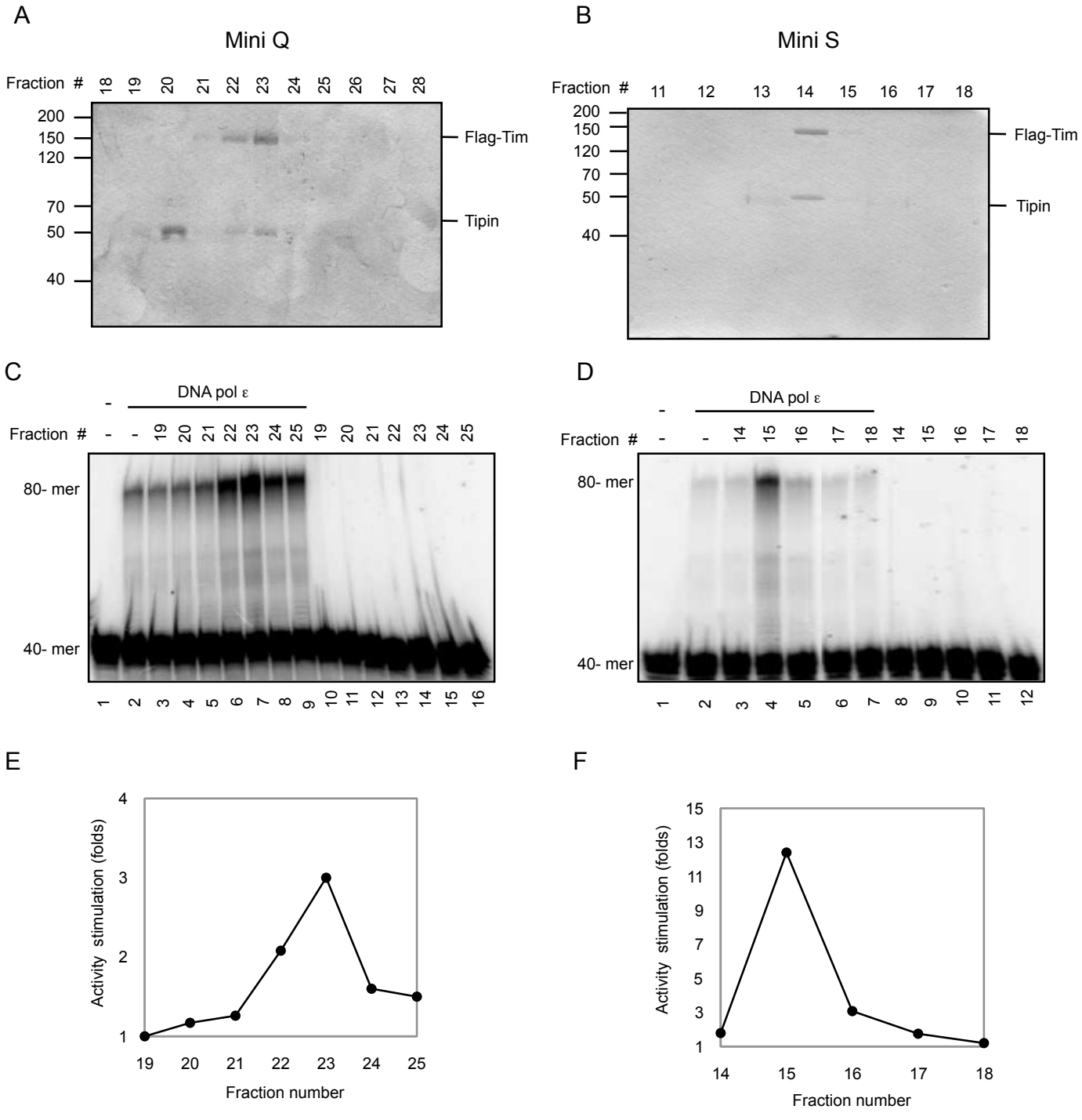
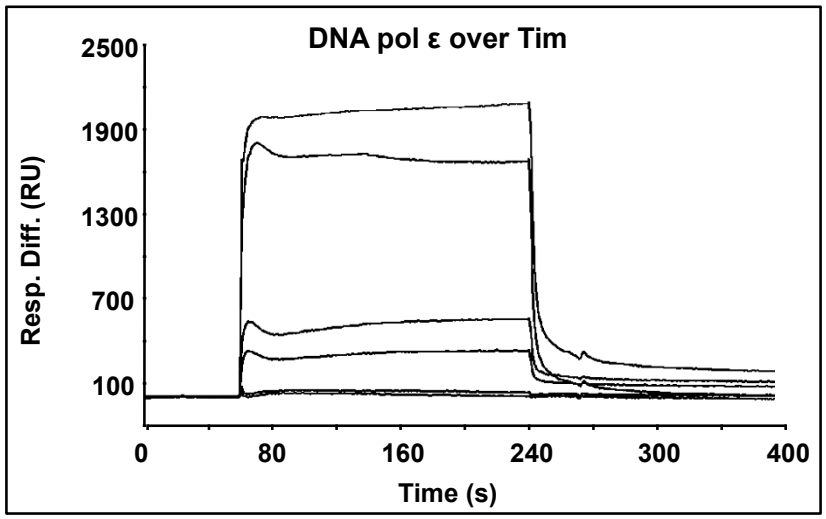


Fig. 5

A



B

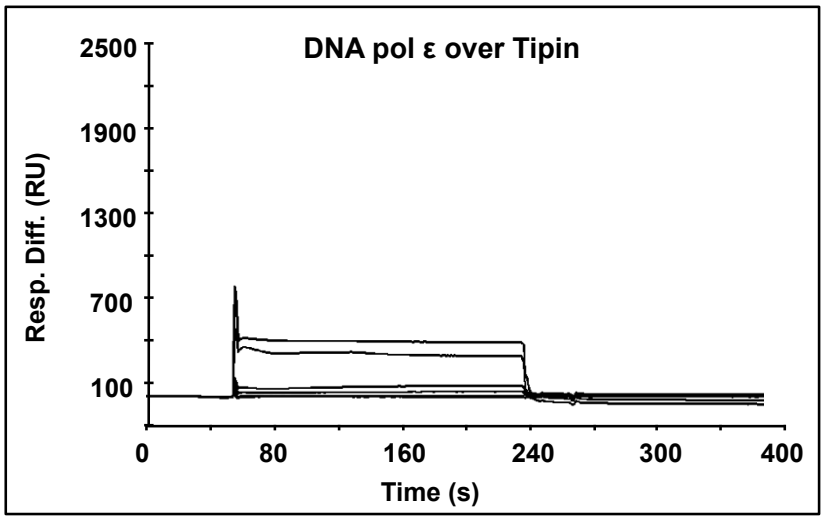


Fig. 6

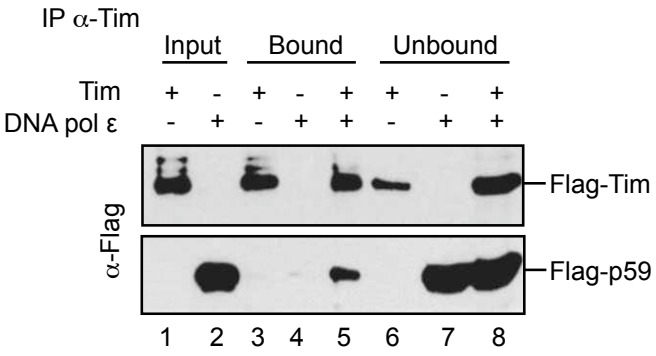


Fig. 7

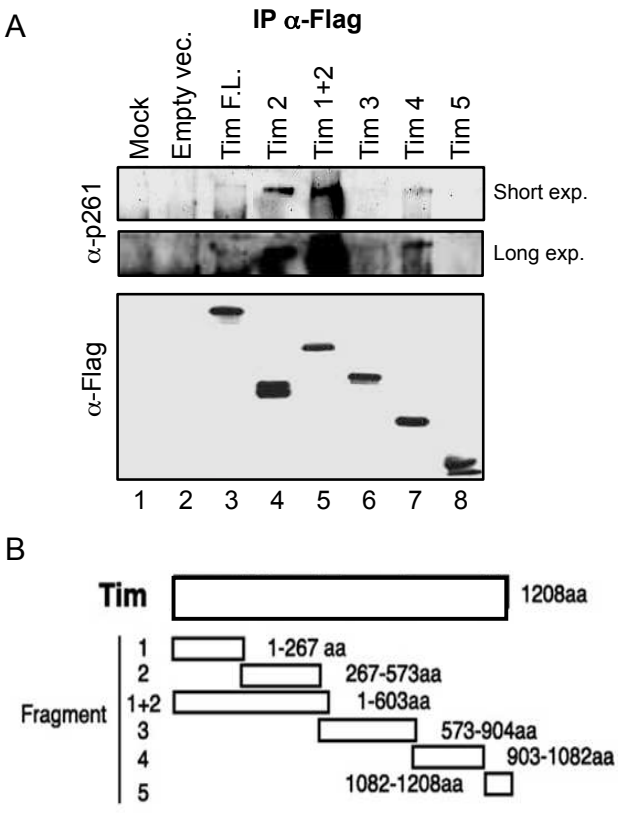


Fig. 8

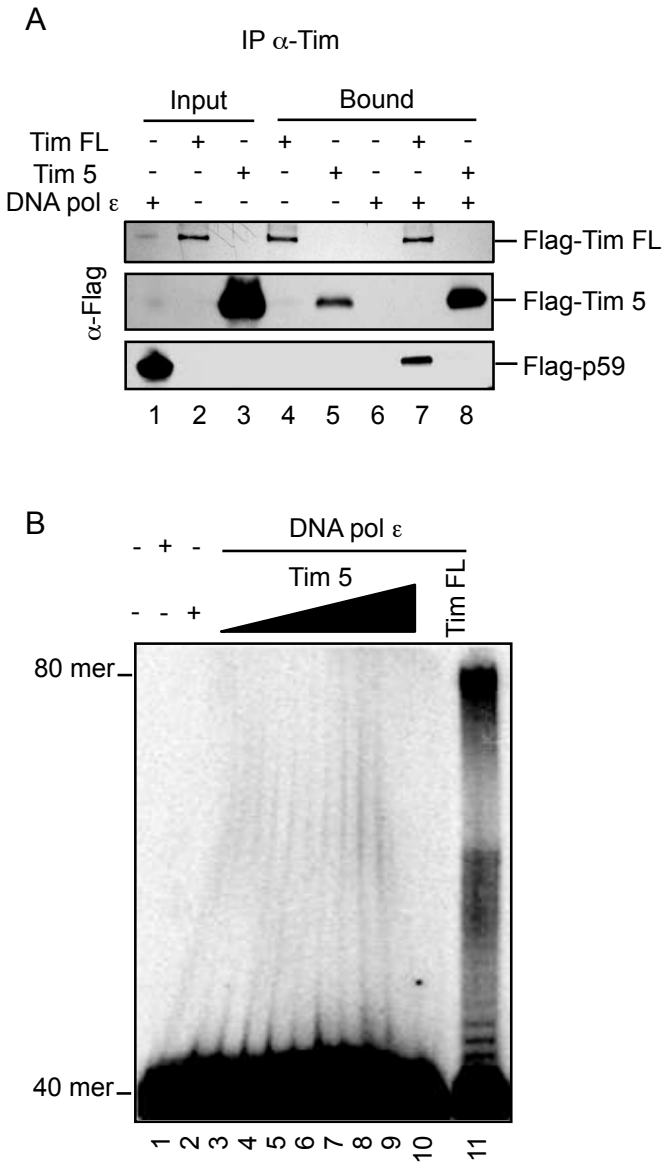


Fig. 9